Particulate–Cell Interactions and Pulmonary Cytokine Expression

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The type II cell plays an important role in the response of the alveolar epithelium after lung injury through its synthesis and secretion of pulmonary surfactant, and by acting as the stem cell for the replacement of damaged type I epithelial cells. The nonciliated bronchiolar epithelial (Clara) cell is thought to play a similar role during repair of the bronchiolar epithelium. Recent evidence has suggested that epithelial cells may participate in aspects of the inflammatory response and regulation of fibroblast growth during pulmonary fibrosis through the production of and response to specific growth factors and cytokines. The cellular and molecular responses of epithelial cells and how they lead to the progression of events that defines the pulmonary parenchymal response to a class of particles is unclear. We used particles differing in size, chemical composition, and fibrogenicity *in vivo* and *in vitro* to elucidate early changes in proinflammatory and profibrotic cytokine and antioxidant gene expression in lung cells. Early increases in mRNA and protein for the proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha have been observed in epithelial cells following exposure. These are accompanied by changes in specific epithelial genes including surfactant protein C and Clara cell secretory protein. The data indicate that effects on the epithelium are due to direct interactions with particles, not a result of macrophage-derived mediators, and suggest a more significant role in the overall pulmonary response than previously suspected. These results suggest that type II cell growth factor production may be significant in the pathogenesis of pulmonary fibrosis. — *Environ Health Perspect* 105(Suppl 5):1179–1182 (1997)

Key words: lung, epithelium, inflammation, particulate, cytokine, TNF-α, IL-1β, IL-6

Introduction

The pulmonary response to inhaled particulates has been the subject of comprehensive investigation for many years. Studies of the deposition and clearance of such inhaled materials provide a useful framework for a more in-depth study of the cellular and molecular events that are involved in the acute and chronic manifestations of particle-induced lung injury (*1*). Much of the initial phase of such studies investigated physiologic aspects of the pulmonary response to particles including investigations of phagocytosis, development of markers of acute injury, and quantitation of inflammatory cell recruitment (*2*). More recent investigations attempt to address aspects of the ability of various inhaled materials to influence gene expression in specific lung cell populations and the relationship of alterations to previously characterized inflammatory changes (*3,4*). Additionally, these studies attempt to provide mechanistic information relating these inflammatory gene changes and the progression of this acute response to a chronic disease state such as fibrosis or the induction of pulmonary carcinogenesis (*5–10*).

Most studies of this type address the initial phase of injury, the inflammatory response, and focus on the expression of inflammatory cytokine genes and their temporal relationship to the recruitment of inflammatory cells by the lung (*5–10*). The overwhelming majority of these studies investigated the production of cytokines and other aspects of altered gene expression by the alveolar macrophage (*11–13*). Such studies show the remarkable ability of the resident alveolar macrophage to upregulate its expression of a wide variety of proinflammatory (*10,14–16*) and profibrotic (*17,18*) cytokines.

Investigations of the pulmonary epithelium response following particle exposure have been less well studied. Pulmonary epithelial cells have been extensively studied with regard to their functions as the producers of pulmonary surfactant and their role in the metabolism of inhaled and systemic lipophilic substances (*19*). More recently, a number of studies suggested that these epithelial populations may also play a role in defining the inflammatory environment within the lung. Of particular interest in this study is the observation that the pulmonary epithelial cells can produce a variety of inflammatory cytokines [for review, see Simon and Paine (*20*)]. Expression of granulocyte macrophage-colony-stimulating factor, tumor growth factor (TGF)-β (*21*), and TGF-α (*22*) have been detected in type II cells. More directly related to the question of inflammatory cell recruitment is the production of the specific chemoattractants interleukin (IL)-8 (*23,24*) and regulated on activation normal T cell expressed and secreted (*25*) by alveolar epithelial cells and cell lines *in vivo* and *in vitro*. In addition, studies show increased expression of macrophage inflammatory protien (MIP)-1α, MIP-2, and tumor necrosis factor (TNF)-α following mineral dust exposure (*16,26*).

This study expands on this aspect of the pulmonary response to particles by examining the expression of proinflammatory cytokines in the lungs of rats and mice following exposure to particles of varying size and chemical composition, in relation

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Abbreviations used: Clara, nonciliated bronchioepithelial; IL, interleukin; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; PTFE, polytetrafluoroethylene; TGF, tumor growth factor; TNF, tumor necrosis factor.

to their ability to induce acute and chronic lung injury.

Materials and Methods

Tissues and fluids used for cytokine analysis were derived from C57Bl/6J mice exposed to TiO_2 and Ni_2S_3 by inhalation, as described previously, and sacrificed at the times indicated in the figure legends. Measurement of acute increases in lavage and plasma IL-6 and TNF were carried out in fluids obtained from C57Bl/6J mice exposed to ultrafine polytetrafluoroethylene (PTFE) fumes, as described previously by Johnston et al. (*27*). These animals were sacrificed 6 hr after a 30-min exposure to 1.25, 2.5, or 5×10^5 particles/cm^3 or sham controls. Lipopolysaccharide (LPS)-injected mice were sacrificed 2 hr after intratracheal or intraperitoneal administration of 10 µg LPS (Sigma Chemical, St. Louis, MO).

Lavage fluid was collected from mice sacrificed by pentabarbital overdose; saline at a volume of 1 ml for 10 repeated lavages was used. Plasma samples were collected by cardiac puncture prior to lavage and anticoagulated with a fixed volume of heparin (50 µl).

Cytokine analysis was carried out by use of specific murine cytokine enzyme-linked immunosorbent assay kits purchased from Biosource International, Camarillo, CA and by following the manufacturer's recommended protocol. *In situ* hybridization for epithelial markers was performed as described previously (*27*).

Results and Discussion

Figures 1 and 2 show changes in the cytokines IL-6 and TNF in plasma and lavage fluid following both intratracheal and intraperitoneal administration of LPS. It is clear that exposure to this potent inducer of inflammation caused a dramatic increase in cytokine abundance in both the plasma and lavage compartments. When LPS was delivered directly to the lung, IL-6 was significantly elevated in both lavage and plasma compartments. In contrast, systemic LPS led to a much greater plasma response than that measured in the pulmonary compartment. This would suggest that measurement of a plasma/lavage ratio for this cytokine may be useful in determining the source of the inflammatory stimulus.

This is further reinforced by the TNF data shown in Figure 2. Localization of this cytokine appears restricted to the compartment where the stimulus was introduced. Virtually no crossover between lung and plasma compartments was observed when TNF was measured. This suggests the importance of locally generated cytokines in the regulation of pulmonary inflammation, especially as related to the role of TNF in the regulation of the production of other cytokines and chemotactic factors.

Nevertheless, these studies show the utility and sensitivity of cytokine analysis of murine lung lavage in the characterization of the regulation of the acute phase of pulmonary injury.

Figure 3 shows the absolute change in lavage IL-6 amounts following acute PTFE fume inhalation. In a previous study (*27*) we showed that acute PTFE fume inhalation leads to an increase in mRNA for IL-6. At the highest exposure level this was nearly 40-fold. Measurement of IL-6 protein suggests a threshold response to these particles with no increase noted at the lowest exposure concentration and a greater than 100-fold increase in protein at the higher particle concentrations. Analysis of TNF-α and IL-β in the same lavage fluid showed a more modest 2-fold increase in these cytokines in the highest particle exposure group. Plasma cytokine measurements showed no significant increase in any of these cytokines, which implicates the pulmonary compartment as the site of cytokine synthesis in this model.

Both sets of previous experimental data show the ability and utility of measuring cytokines, namely IL-6, following acute lung injury associated with a profound inflammatory response. The data in Figure 4 suggest that IL-6 can also be used as a marker for pulmonary response in an inflammatory process as induced during chronic particle inhalation. Lavage fluid obtained from mice exposed to the nontoxic

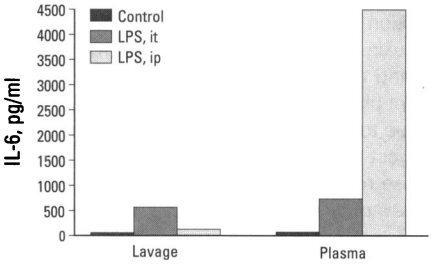

Figure 1. Measurement of IL-6 following lipopolysaccharide administration. Bronchoalveolar lavage fluid and plasma samples were collected from mice 2 hr after intratracheal (it) or intraperitoneal (ip) administration of LPS as described in "Materials and Methods." Immunoreactive IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) using monospecific antibodies. Results represent the mean of triplicate determinations of samples collected from four individual animals. Interassay variability was less than 5%.

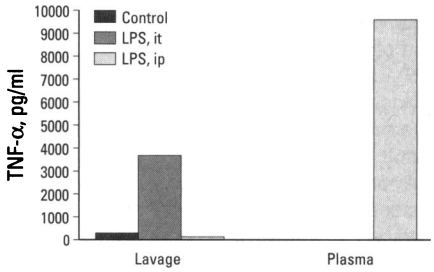

Figure 2. Measurement of TNF-α following lipopolysaccharide administration. Bronchoalveolar lavage fluid and plasma samples were collected from mice 2 hr after intratracheal or intraperitoneal administration of LPS as described in "Materials and Methods." Immunoreactive IL-6 was measured by ELISA using monospecific antibodies. Results represent the mean of triplicate determinations of samples collected from four separate animals. Interassay variation between samples was less than 5%.

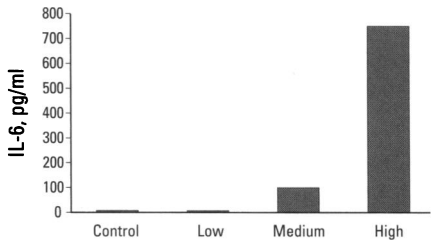

Figure 3. Measurement of IL-6 following polytetrafluoroethylene fume inhalation. Bronchoalveolar lavage fluid was obtained from mice 6 hr after a 30-min exposure to PTFE fumes at high (5×10^5), medium (2.5×10^5), and low (1.25×10^5) particles/cm^3, respectively. IL-6 was assayed by ELISA. Data represent the mean of duplicate determinations of samples from three animals.

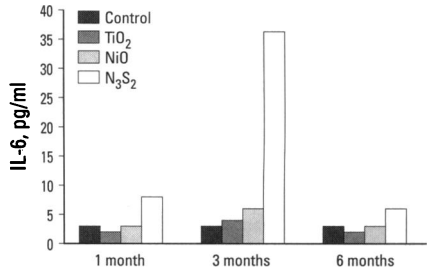

Figure 4. Measurement of IL-6 following chronic particle inhalation. Bronchoalveolar lavage fluid was obtained from mice at the indicated times during a 13-week exposure at 6 hr/day, 5 days/week of 10 mg/m^3 TiO_2-F, 5 mg/m^3 NiO, or 0.5 mg/m^3 Ni_3S_2. IL-6 was assayed by ELISA. Data represent the mean of duplicate determinations of four separate samples for each particle group. Abbreviation: TiO_2-F, standard (200 nm diameter).

particle TiO_2 (200 nm diameter) and two suspected pulmonary carcinogens NiO and Ni_2S_3 were analyzed for IL-6. Although all three particles caused some increase in inflammation markers, these were significant only for the Ni_2S_3 group (data not shown). Total lavage cells increased nearly 10-fold, with lymphocytes and granulocytes increasing 30- and 2-fold, respectively. This inflammation is clearly reflected in the amount of IL-6 detected in the lavage. Although the magnitude of the change is not nearly as dramatic as seen in the acute injuries, there was a clear association between pulmonary inflammation and detectable lavage IL-6.

Although the data presented suggest measurement of IL-6 can reflect the inflammatory state of the lung, none of these changes indicate the cell population that is responsible for its production. Although it is logical to assume that the production of this and other cytokines is due to the activation of the recruited inflammatory cells, we evaluated the hypothesis that particle-induced inflammation could induce the expression of a cytokine subset by pulmonary epithelial cells.

We initiated a series of studies designed to measure the ability of isolated type II cells to produce inflammatory cytokines *in vitro*. Figures 5 and 6 show the results of the incubation of cultured primary type II cells with various forms and concentrations of TiO_2. Consistent with its more potent inflammatory response *in vivo*, Figure 5 shows that addition of 10 µg/ml ultrafine TiO_2 (10 nm diameter) to cultured type II cells leads to increased release of TNF. This increase persisted throughout the entire 48-hr incubation period. In contrast, a standard TiO_2 particle (200 nm diameter) elicited no cytokine increase. A similar result is evident in Figure 6, where IL-1 production following culture with TiO_2 (10 nm diameter) is illustrated.

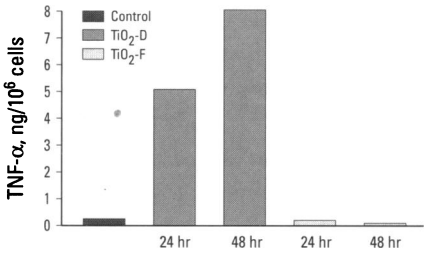

Figure 5. Measurement of TNF-α release by primary isolated type II cells in culture. Type II cells were cultured at $10^5/cm^2$ for 24 hr in DME/F12 medium. At that time media was removed and replaced with serum-free media containing the indicated particle at 10 µg/ml. After the indicated interval, media was removed and assayed for TNF by ELISA. Results represent the mean of triplicate meaurements of two separate cultures of cells. Abbreviation: TiO_2-D, ultra fine (10 nm diameter).

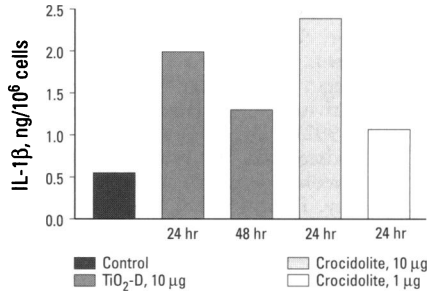

Figure 6. Measurement of IL-1 release by primary isolated type II cells in culture. Type II cells were isolated from adult mouse lungs by standard protease digestion and cultured at $10^5/cm^2$ for 24 hr in DME/F12 medium. At that time media was removed and replaced with serum-free media containing the indicated particle at 10 µg/ml. After the indicated interval, media was removed and assayed for IL-1 by ELISA. Results represent the mean of triplicate meaurements of two separate cultures of cells.

Type II cell cytokine expression following *in vivo* particle exposure was measured by a series of studies using *in situ* hybridization to localize cytokine mRNA. As a preliminary step we established the precise localization of pulmonary epithelial population in the lung through the use of epithelial-specific markers. We chose nonciliated bronchioepithelial (Clara) cell secretory protein and surfactant apoprotein C as markers for Clara and type II cells, respectively. Our initial data suggest that not only do type II cells show increased abundance of cytokine mRNA (*27*), but acute and chronic pulmonary inflammation lead to alterations in epithelial marker gene expression. We believe changes in such marker genes, in the absence of specific morphological evidence of cytotoxicity and epithelial damage, indicate a change in the activation state of the epithelium and may act as a marker for increased cytokine gene expression. Studies of type II cell activation and hypertrophy following instillation of crystalline silica (*28,29*), and recent work to examine changes in surfactant apoprotein gene expression following LPS administration, suggest alterations in epithelial gene expression are closely linked to inflammatory changes in the lung (*30*). The relationship between such expression and the pulmonary production of cytokines requires closer examination. Recent studies suggest that surfactant apoprotein gene expression can be altered in response to elevated TNF (*31*). Thus a more careful examination of the temporal sequence of cytokine and epithelial markers is necessary. This is especially true if expression of such epithelial markers will be used to identify a specific epithelial cell population as the site of cytokine gene expression.

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